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PD-L1 expression in endometrial carcinoma cells and intratumoral immune cells: differences across histological and TCGA-based molecular subgroups

Annukka Pasanen MD[1,2], Terhi Ahvenainen MSc[3,4], Teijo Pellinen PhD[5], Pia Vahteristo PhD[3,4], Mikko Loukovaara MD, PhD[6], Ralf Bützow MD, PhD[1,2,6]

[1]Department of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.
[2]Applied Tumor Genomics Research Program, University of Helsinki, Helsinki, Finland
[3]Department of Medical and Clinical Genetics, Medicum, University of Helsinki, Helsinki, Finland.
[4]Genome-Scale Biology Research Program, Research Programs Unit, University of Helsinki, Helsinki, Finland.
[5]Institute for Molecular Medicine Finland (FIMM), Helsinki, Finland.
[6]Department of Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.

Corresponding author:
Annukka Pasanen, Department of Pathology, University of Helsinki and Helsinki University Hospital, Haartmaninkatu 3, 00290 Helsinki, Finland. Electronic address: annukka.pasanen@hus.fi

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Abstract

PD-L1 is a biomarker that may predict the response to antiPD-1/PD-L1 immunotherapy. We evaluated the expression of PD-L1 in carcinoma cells and immune cells across histopathological and TCGA molecular subgroups of endometrial carcinoma.

Our study included 842 patients with endometrial carcinoma. Direct sequencing of polymerase epsilon (*POLE*) exonuclease domain hot spots and conventional immunohistochemistry (MLH1, PMS2, MSH2, MSH6, p53) were conducted to identify TCGA classification-based molecular subgroups of endometrial carcinoma: *POLE*-mutated, mismatch repair (MMR) deficient, no specific molecular profile and p53-aberrant. Multiplex immunohistochemistry was performed to evaluate PD-L1 expression in carcinoma cells (Ca) and tumor-infiltrating immune cells (ICs). PD-L1 expression in carcinoma cells and in ICs was detected in 8.6% and 27.7% of the cases, respectively. Combined positive score (CPS) was ≥1% in 19.4% of the samples. PD-L1 positivity in carcinoma cells, ICs and CPS correlated with tumor T cell density (TILs, $p<0.001$). *POLE*-mutated and MMR-deficient tumors were more likely to present PD-L1 expressing ICs, CPS positivity and abundant TILs compared with other TCGA subgroups ($p<0.001$). No differences existed in Ca-PD-L1 expression ($p=0.366$). Within various histotypes, non-endometrioid carcinomas displayed the highest Ca-PD-L1, ICs and CPS ($p<0.03$). Advanced cancers showed more frequent Ca-PD-L1 positivity ($p=0.016$), CPS ($p=0.029$) and IC≥1% ($p=0.037$) positivity compared to early disease.

In conclusion, PD-L1 expression profiles differ between molecular subclasses, histological subtypes and disease stage of endometrial carcinoma. Prospective studies are needed to explore the predictive value of various PD-L1 scoring systems within the subgroups of endometrial cancer. CPS presents methodological advantages over cell-type specific scoring systems.

Key words: endometrial carcinoma, PD-L1, TCGA classification

INTRODUCTION

Immune checkpoint inhibitors have emerged as a promising treatment option for various types of cancer, but their potential in endometrial cancer (EC) is unknown (1). Immunotherapy enhances the immune system's innate potential to fight cancer cells. An effective anti-tumor response relies primarily on the capability of T cells to recognize tumor-derived peptides as non-self. Typically, these immunogenic peptides are produced as a consequence of mutations and, accordingly, tumors with a high mutational burden are presumed to be highly immunogenic. Immune responses are controlled by various pathways that tumor cells may exploit to escape immune surveillance. One of the main immunosuppressive pathways is the PD-1/PD-L1 interaction taking place between T cell programmed death 1 (PD-1) receptor and its ligand PD-L1 located on various types of cells, including immune cells and carcinoma cells (2). Antibody-mediated blockade of the PD-1/PD-L1 immune checkpoint has been shown to reverse T-cell inactivation exposing tumor cells to immune attack (3). Anti-PD-1/PD-L1 therapies have produced impressive treatment outcomes in patients with non-small cell lung cancer, melanoma and renal cell carcinoma (1,4). Limited-sample studies have obtained promising results also with endometrial carcinoma patients (5,6). Also other immune checkpoints (e.g. CTLA-4, LAG-3) may induce immunosuppression in tumors (7,8).

Patients tolerate immunotherapy well compared to standard chemotherapy, but side effects may occur. The therapy is costly and not all patients benefit from it. Thus, an efficient use of immunotherapy requires appropriate patient selection strategies. Often the selection of patients to anti-PD-1/PD-L1 therapy is guided by PD-L1 immunohistochemical (IHC) assays. Scoring methods and adopted cut-offs vary according to the tumor type and individual anti-PD-1/PD-

L1 agents. Proposed scoring algorithms evaluate PD-L1 positivity in carcinoma cells and/or immune cells separately or in combination (combined positive score, CPS) (9-11). Reported frequencies of PD-L1 positivity in endometrial carcinoma vary considerably (0.9-44.3%) even in unselected EC cohorts (Table 1) (12-24). Such variability may in part derive from different antibody clones and different cut-offs. In fact, notable interassay variation has been reported within commercially available PD-L1 immunohistochemical assays (25,26). Accuracy of IHC scorings may also suffer from problems related to traditional chromogenic PD-L1 immunohistochemistry. Staining of the tumor cells may be weak and unspecific cytoplasmic staining occurs. Moreover, intratumoral T cells and macrophages often present membranous staining and they may be misinterpreted as carcinoma cells (27). Multiplex IHC overcomes these limitations by simultaneous detection of a biomarker and numerous cell-specific markers on a single paraffin tissue section, allowing the identification and quantification of various cell types expressing the antigen of interest (28).

Endometrial carcinoma is not a uniform disease entity, as it comprises various histological and molecular subgroups, each with their own clinicopathological characteristics. Given this heterogeneity, exhaustive biomarker studies rely on well-powered subclass analyses. The goal of our study was to explore PD-L1 expression and T cell inflammation within histological subtypes and TCGA-based molecular subgroups of endometrial cancer. Fluorescent multiplex immunohistochemistry was performed to overcome limitations related to traditional immunohistochemical evaluation.

MATERIALS AND METHODS

Patients who underwent primary surgical treatment for endometrial cancer at the Department of Obstetrics and Gynecology, Helsinki University Hospital, between January 1, 2007, and

December 31, 2012, were identified (n = 965). Patients with adequate tumor samples for a tissue microarray (TMA) were included in the study (n = 842). Approvals of the Institutional Review Board and the National Authority for Medicolegal Affairs of Finland were obtained. Clinical data were collected from institutional medical records. Lacking follow-up data were obtained from Statistics Finland or completed by contacting primary physicians at the referring institutions.

We performed immunohistochemistry on multicore tissue microarray (TMA) slides, prepared as described before (29). The following monoclonal antibodies were used for chromogenic immunohistochemistry: MLH1 (ES05, Dako), PMS2 (EPR3947, Epitomics), MSH-2 (G219-1129, BD Biosciences), MSH-6 (EPR3945, Abcam), p53 (DO-7, Dako) and PD-L1 (SP263, Ventana). TMA slides were scanned with 3-dimensional Histech Pannoramic 250 Flash II scanner by Fimmic Oy (Helsinki, Finland). Slide images were managed and analyzed with WebMicroscope Software (Fimmic Oy). Virtual slides were scored by a pathologist blinded to clinical data. A second investigator examined equivocal cases and a consensus was reached. Mismatch repair protein status was considered deficient (MMRd) when we observed a complete loss of nuclear expression in carcinoma cells of one or more MMR proteins (MLH1, PMS2, MSH2, MSH6) detected by immunohistochemistry. Aberrant p53 staining was defined as strong and diffuse nuclear staining or completely negative ('null') staining in carcinoma cells. Weak and heterogeneous staining was classified as wild type expression. Stromal cells and inflammatory cells served as internal control for MMR and p53 stainings. Samples with scarce carcinoma cells or with completely negative staining of the internal control (when applicable), were discarded. Representative images of MLH-1 and p53 staining patterns are shown in Figure 1.

The fluorescent multiplex immunohistochemistry was carried out as described by Blom et al. with following modifications (30). Primary antibodies were: PD-L1 (CST, E1L3N), CD3

(Thermo, MA5-14482), CD163 (Abcam, ab188571), and PanEpi (cocktail of anti-PanCk, C-11, Abcam, Ab77531; anti-PanCk AE1/AE3, InVitrogen, 180132; E-cadherin, BD clone 36). Nuclei were stained using DAPI (Roche). Five-channel fluorescent images were acquired using Metafer 5 scanning and imaging platform (MetaSystems, Alltlussheim, Germany) equipped with AxioImager Z2 microscope with a 20x objective (Carl Zeiss, Goettingen, Germany) and a CoolCube 2m CCD camera (MetaSystems, Alltlussheim, Germany). The image analysis was carried out both visually by a pathologist and by a cell image analysis software (CellProfiler version 2.2.0). Scoring was primarily performed by a pathologist and in rare equivocal cases automated image analysis was used to support the decision-making. Necrotic areas and scarce samples (<100 cells) were excluded from scoring. PD-L1 expression was defined as partial or complete membranous staining in carcinoma cells and membranous and/or cytoplasmic staining in immune cells (CD3-positive T lymphocytes and CD163-positive macrophages within tumor nests and/or adjacent supporting stroma). We determined the percentage of positive carcinoma cells and immune cells separately and in combination. To calculate the combined positive score (CPS), we divided the total number of PD-L1-positive cells (carcinoma cells, lymphocytes, and macrophages) by the number of viable carcinoma cells, multiplied by 100 (9). Semiquantitative scoring was adopted as follows: 0: <1% of the cells; 1: 1-4%; 2: 5-9%; 3: 10-49%; 4: ≥50%. The cut-off for positive PD-L1 staining was set at 1%. The cut off for strong positivity was set according to the results of a previous randomized trial (≥50% for carcinoma cells and ≥10% for immune cells) (31). Comparative images of conventional chromogenic immunohistochemistry and multiplex immunofluorescence of PD-L1 positive and negative cells are shown in Figure 2. Tumoral CD3+ lymphocytic infiltration (TILs) was semi-quantitatively scored as scarce, moderate or abundant.

For DNA extraction, representative areas of formalin-fixed paraffin-embedded tumor tissue were macrodissected as identified by pathologist assessment. DNA was extracted by proteinase

K/phenol-chloroform method. *POLE* exonuclease domain mutation screening of hot spots in exon 9 (c.857C>G, p.P286R; c.890C>T, p.S297F), exon 13 (c.1231G>C, p.V411L) and exon 14 (c.1366G>C, p.A456P), was performed by direct sequencing. The following primers were used: Ex 9F (5'-3'): CCTAATGGGGAGTTTAGAGCTT; Ex 9R (5'-3'): CCCATCCCAGGAGCTTACTT; Ex 13F (5'-3'): TCTGTTCTCATTCTCCTTCCAG; Ex 13R (5'-3'): CGGGATGTGGCTTACGTG; Ex 14F (5'-3'): TGACCCTGGGCTCTTGATTT; Ex 14R (5'-3'): ACAGGACAGATAATGCTCACC. PCR products were sequenced on an ABI3730xl Automatic DNA Sequencer at Institute for Molecular Medicine Finland (FIMM), Helsinki. Sequence graphs were analyzed both manually and with Mutation Surveyor (Softgenetics, State College, PA). Only cases with good-quality sequence for all the examined *POLE* hot spots were included in the analysis.

Pearson χ^2 test and Fisher exact test were used for comparisons of categorical variables. Survival curves were calculated by the Kaplan-Meier method. A log-rank test was used to test for survival differences. Disease-specific survival was defined as the time from date of surgery to death from endometrial cancer. Statistical significance was set at $p < 0.05$. Cohen's kappa statistics were calculated to measure the agreement between multiplex and chromogenic immunohistochemistry for PD-L1. Based on kappa references outlined by Landis and Koch, the strength of agreement was considered fair for kappa values between 0.21 and 0.40 and moderate for kappa values between 0.41 and 0.60 (32). Data were analyzed using IBM SPSS version 25 software (IBM Corp., Armonk, New York, USA).

RESULTS

Clinicopathological characteristics of the study cases are summarized in Table 2. Of the 842 patients included in the study, 745 (88.5%) had endometrioid and 97 (11.5%) non-endometrioid carcinoma. Median follow-up of patients was 78 months (range 1 to 136 months). Sequencing

of all the targeted genomic regions of *POLE* was successful for 553 cases. *POLE* mutation was detected in 7.4% of endometrioid carcinomas and 4.0% of non-endometrioid carcinomas (6.7% of all cases). MMR protein deficiency was found in 37.7% of endometrioid carcinomas and 25.6% of non-endometrioid carcinomas (36.2% of all the cases). Aberrant p53 profile was detected in 10.8% of endometrioid tumors and 61.9% of non-endometrioid tumors (16.8% of all the samples). A minority of cases displayed multiple molecular features. Both *POLE* mutation and aberrant p53 expression were present in 0.4% of the cases and both *POLE* mutation and MMR protein deficiency in 0.2% of the patients. Only one sample (0.2%) had all three molecular alterations. These patients were allocated into the *POLE*mut molecular subgroup. Both MMR deficiency and aberrant p53 status were detected in 3.1% of the cases. These were classified as MMRd tumors.

In the multiplex immunofluorescence staining, 8.6% of the cases presented PD-L1 expression on carcinoma cells (Ca≥1%) and 27.7% on intratumoral immune cells (ICs≥1%). CPS was positive (CPS≥1%) in 19.4% of the samples. High PD-L1 expression (Ca≥50% or ICs≥10%) was observed in 0.5% and 8.6% of the cases, respectively. Relative frequencies of semiquantitative staining scores are presented in Table 3. Tumors with moderate-abundant T cell density presented PD-L1 positivity in carcinoma cells (10.6%), ICs (36.6%) and CPS (26.8%) more frequently than tumors with scarce lymphocytic infiltration (Ca 5.6%, p=0.019; ICs 14.6%, p<0.001, CPS 8.4%, p<0.001). Concomitant presence of moderate-abundant T cell infiltrates and any PD-L1 positivity ("T cell inflamed PD-L1 positive" phenotype), was observed in 25.1% of all the tumors.

Relative frequencies of PD-L1 positivity in carcinoma cells varied significantly between histotypes: endometrioid carcinoma 8.0%, clear cell carcinoma 14.7%, serous carcinoma 3.7%, undifferentiated carcinoma 14.7% and carcinosarcoma 20% (p=0.022, Figure 3). Observed relative frequencies of CPS≥1% were: endometrioid carcinoma 17.1%, clear cell carcinoma

38.2%, serous carcinoma 37.0%, undifferentiated carcinoma 42.9% and carcinosarcoma 26.7% ($p<0.001$; Figure 3). Similar differences were noted in the immune cell expression of PD-L1 with significantly higher relative frequencies of expression in the non-endometrioid carcinomas ($p=0.028$). By contrast, we found no statistically significant differences between histological subgroups and strong PD-L1 positivity in ICs ($p=0.148$, Figure 3). Our cohort included only one neuroendocrine carcinoma, which presented PD-L1 expression on both carcinoma cells and immune cells. We found no correlation between Ca-PD-L1 expression and grade of differentiation of endometrioid carcinomas (G1-2 vs G3, $p=0.08$), whereas CPS and IC≥10% PD-L1 expression were more frequent in G3 as compared to G1-2 endometrioid carcinomas (33.0% vs 14.3% and 20.8% vs 5.4%, respectively, $p<0.001$). The overall quantity of CD3+ TILs (scarce-moderate vs abundant) did not differ significantly in histological subgroups ($p=0.158$) or between grade of differentiation of endometrioid carcinoma ($p=0.722$).

PD-L1 expression profiles were also analyzed according to FIGO 2009 stage of disease (stage I-II vs III-IV, Figure 3). Samples from patients with advanced stage (III-IV) disease were more likely to present Ca-PD-L1 positivity (13.6% vs 7.5%, $p=0.016$), CPS (25.9% vs 18.0%, $p=0.029$) and IC≥1% (34.7% vs 26.2%, $p=0.037$) positivity as compared to early stage (I-II) disease. Differences in the IC≥10% ($p=0.270$) or the overall quantity of TILs ($p=0.598$) were not statistically significant. In advanced disease, strong Ca-PD-L1 positivity was found in 1.4% of the cases and strong IC positivity in 10.9% of the cases.

Samples with successful *POLE* sequencing and immunohistochemical stainings of MMR proteins and p53 (512 cases), were stratified into TCGA-based molecular subclasses. *POLE*mut and MMRd tumors exhibited higher relative frequencies of immune cell PD-L1 positivity (55.9% and 40.9%) and CPS positivity (44.1% and 29.6%) compared to NSMP (IC: 13.9%, CPS: 9.1%) and p53ab cases (25.4%, 20.9%; $p<0.001$, Figure 4). Significant differences were observed also for strong positivity in ICs ($p<0.001$, Figure 4). *POLE*mut and MMRd cases were

also more likely to present abundant intratumoral T cell infiltrates (26.5% and 27.8% respectively) compared to NSMP and p53ab cases (15.3% and 16.7% respectively; p=0.014). Similarly, we observed “T cell inflamed PD-L1 positive” phenotype more frequently in *POLE*mut and MMRd groups (50.0% and 34.9%, respectively) compared to other TCGA subclasses (16.3% and 17.9%; p<0.001). PD-L1 expression in carcinoma cells showed no correlation with TCGA classification (p=0.366).

In Kaplan Meier analysis, disease specific survival segregated by histotype and TCGA subgroups as expected (p≤0.001, data not shown). *POLE*mut group had excellent outcomes (no disease related deaths in this group) and aberrant p53 status associated with poor disease specific survival. Scarce overall quantity of TILs predicted poor prognosis (p =0.001), whereas PD-L1 expression on carcinoma cells, ICs or CPS showed no correlation with outcome (p=0.298, p=0.592, p = 0.569, respectively).

According to kappa statistics, multiplex and chromogenic immunohistochemistry scorings showed moderate agreement for CPS (kappa 0.540) and poor agreement for PD-L1 expression in carcinoma cells (kappa 0.279).

DISCUSSION

In the evolving era of personalized medicine, immunotherapy offers new treatment options for cancer patients. FDA has approved mismatch repair deficiency/microsatellite instability as selection criteria for anti PD-1/PD-L1 therapy (33). Treatment indications in mismatch repair stable EC and the role of biomarkers, including PD-L1, have remained unsettled. To facilitate prospective studies, we profiled PD-L1 expression across histopathological and TCGA molecular subgroups of endometrial carcinoma.

In our study cohort, 8.6% of the cases presented PD-L1 expression in carcinoma cells and 27.7% in the ICs. In line with previous studies, PD-L1 expression on carcinoma cells or on lymphocytes showed no correlation with survival (18,19). Non-endometrioid carcinomas were more likely to present PD-L1 positive carcinoma cells, CPS and ICs compared to endometrioid carcinomas. In the subgroup of endometrioid ECs, high grade of differentiation was associated with more frequent CPS and IC positivity compared to low grade disease.

In a landmark study, The Cancer Genome Atlas (TCGA) identified 4 distinct molecular subgroups of endometrial carcinoma: *POLE* ultramutated, microsatellite instability hypermutated (MSI-H), copy-number-low microsatellite stable (MSS), and copy-number-high (34). Vast majority (90%) of the copy-number-high tumors presented TP53 mutations. Consequently, TP53 mutational analysis or immunohistochemical analysis of p53 expression have been proposed as a surrogate marker for this subgroup of tumors (35,36). *POLE* mutated tumors are characterized by defects in the proof-reading function of DNA polymerase epsilon and harbor the highest rate of somatic mutations, followed by MSI-H tumors characterized by defects in DNA mismatch repair activity. These highly mutated tumors have been reported to contain a large number of predicted neoantigens and activated cytotoxic tumor infiltrating T lymphocytes, often expressing PD-1 and PD-L1 (13,17,24,37,38). Corroborating these findings, we observed significantly higher relative frequencies of heavy T cell infiltrates and PD-L1 expressing ICs in the *POLE* mutated and MMR deficient groups compared to other TCGA subgroups. By contrast, we found no correlation between Ca-PD-L1 expression and the molecular subclasses.

It has been speculated, that tumors most likely to respond to PD-1/PD-L1 blockade characteristically present an “adaptive resistance” phenotype (T cell inflamed PD-L1 positive phenotype, i.e. concomitant presence of intratumoral T cell infiltrates and PD-L1 positivity) (39-41). Consequently, based on previous studies and our results, *POLE*mut and MMRd tumors

become natural candidates for immune checkpoint blockade therapy. Interestingly, in a phase II study of an anti-PD-1 agent in patients with various types of advanced cancer (including endometrial carcinoma), mismatch-repair status itself, and not PD-L1 expression, predicted clinical benefit (42).

Clinical studies suggest a correlation between increasing levels of PD-L1 expression and drug efficacy, but definite scoring systems and cut-offs may be tumor-specific and still need to be determined (43,44). Most trials focus on PD-L1 expression on carcinoma cells. Nonetheless, various studies report associations between clinicopathological characteristics of EC and PD-L1 expression on immune cells rather than tumor cells (13-15,21). The significance of these correlations is unknown. In a trial including multiple cancer types, PD-L1 positivity on tumor-infiltrating immune cells, but not on tumor cells, predicted response to cancer treatment with an anti-PD-L1 agent, MPDL3280A (atezolizumab) (45). Accordingly, for atezolizumab treatment, expression in intratumoral immune cells (IC) is also used as an indicator for potential response (46). In a randomized lung cancer trial, patients with tumors expressing high levels of PD-L1 (defined as Ca≥50% or IC≥10%) derived the greatest benefit from atezolizumab treatment (31). We observed high tumoral Ca-PD-L1 expression in only 0.5% of the tumors. However, strong IC positivity (≥10%) was seen in 8.6% of the cases. The need for alternative treatment options is greatest in advanced stage (III-IV) endometrial carcinoma, which presented with stronger Ca-PD-L1, IC and CPS expression levels than early cancers.

Intratumoral heterogeneity of protein expression may lead to decreased sensitivity in TMA studies. Clonal loss of MMR protein expression has been reported and it is not known whether focal MMR deficiency could invoke a PD-L1 response in a predominantly intact tumor. However, the rate of mismatch repair deficiency in our study was not lower than generally reported in the literature. In a study by Sloan et al., heterogeneous PD-L1 positivity in ECs typically consisted of individual cells or small clusters of cells, that were fairly evenly

distributed throughout the tumor (18). Further, previous studies have shown that TMAs with three core biopsies per tumor adequately represent the tumor phenotype, even with antigens known to be heterogeneous (47,48). Since performing MMR or PD-L1 stainings on whole sections was not feasible for this vast cohort, to improve sensitivity, we included 4 tissue cores from each tumor in our TMA. We have previously demonstrated a high concordance between our TMA and the corresponding whole sections, as concerns expression of L1CAM, a highly heterogeneous antigen (29). As an advantage, TMA methodology allowed us to analyze a large number of cases by multiplex IHC and conventional standardized immunohistochemistry (Ventana clone SP263).

In concordance analysis of multiplex IHC and conventional IHC, carcinoma cell proportion score showed only fair agreement, which in part reflects the difficulty of differentiating PD-L1 positive carcinoma cells from macrophages in the chromogenic IHC. Accordingly, moderate agreement was found between CPS scorings. In some cases, chromogenic immunostainings presented equivocal staining in the stromal compartment, which may have led to false positivity in IC scoring and CPS. Multiplex immunohistochemistry aptly circumvented these limitations. Some of the differences between the staining results may be explained by the low cut off for PD-L1 positivity (1%) and the use of different PD-L1 antibody clones, i.e. E1L3N for multiplex and SP263 for chromogenic IHC. In our experience, multiplex immunohistochemistry clearly outperforms traditional IHC when analyzing PD-L1 expression in various cell types. However, at the moment it cannot be adopted in routine diagnostics and the problems related to cell-type specific scoring systems may be circumvented using a scoring method that combines positivity of both carcinoma and intratumoral immune cells. Based on our results, the correlation of such score (CPS) to clinicopathological characteristics of endometrial carcinoma is equal or better than score based on carcinoma cells only.

In conclusion, we identified differences in PD-L1 expression between histological subtypes, disease stage and TCGA-based molecular subgroups of endometrial carcinoma. PD-L1 positivity was more frequently observed in intratumoral immune cells compared to carcinoma cells. Based on our results, prospective trials should consider not only PD-L1 expression on carcinoma cells but also immune cells, when stratifying patients with endometrial carcinoma for immunotherapy. Combined scoring systems may present methodological advantages over cell-type specific scoring. Further studies are necessary to explore the predictive value of this differential expression of PD-L1, various scoring methods and the applicability of immunotherapy in different subgroups of endometrial cancer.

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453 **LEGENDS**
454

455 Table 1. PD-L1 expression in endometrial carcinoma: overall frequency of PD-L1 positivity (tumoral
456 and immune cells, CPS), cut-offs for positive staining and significant correlations between PD-L1
457 expression and clinicopathological features.
458

459 END=endometrioid, NE=non-endometrioid, MSI=microsatellite instable, MSS=microsatellite stable,
460 MMRd/p=mismatch repair deficient/proficient, Chr=chromogenic immunostaining, CA=carcinoma cells, IC=immune
461 cells, CPS=combined positive score, mut=mutated, LVI=lymphovascular invasion, LN+=lymph nodal metastasis,
462 MI=myometrial invasion; NS=non-significant, PFS=progression-free survival, OS=overall survival; *values extracted
463 from graphs
464
465

466 Figure 1. MLH-1 and p53 immunohistochemistry: a) Endometrial carcinoma cells exhibiting
467 positive nuclear MLH-1 staining, b) Loss of MLH-1 expression in carcinoma cells with
468 tumoral lymphocytes as positive internal control, c) subclonal loss of MLH-1, d) wild type
469 p53, e) aberrant p53 (diffuse overexpression), f) aberrant p53 (null), stromal cells serving as
470 internal control.

471

472 Figure 2. PD-L1 positive (a,b) and negative (c,d) endometrial carcinoma: a,c) PD-L1,
473 chromogenic immunoassay; b, d) Fluorescent multiplexed immunoassay: PD-L1 (blue), EPI
474 (carcinoma cells, white), CD3 (T cells, green), CD163 (macrophages, red). Note the co-
475 localization of PD-L1 and epithelial or immune cell markers: PD-L1 positive carcinoma cells
476 (light blue), lymphocytes (turquoise) and macrophages (magenta)

477

478 Figure 3. Frequency of PD-L1 positivity in carcinoma cells (≥1%), ICs (≥10%) and CPS
479 (≥1%) according to histological subgroups ($p=0.022$, $p=0.148$ and $p<0.001$, respectively) and
480 FIGO 2009 stage ($p=0.037$, $p=0.270$ and $p=0.029$, respectively). Ca=carcinoma cells,
481 ICs=immune cells, CPS=combined positive score

Figure 4. Frequency of PD-L1 positivity in carcinoma cells (p=0.366), ICs (p<0.001), CPS (p<0.001) and presence of heavy T cell infiltrates (p=0.014) according to molecular subgroups. POLEmut = mutated *POLE*, MMRd= MMM deficient, NSMP = no specific molecular type, p53ab = p53 aberrant. Ca=carcinoma cells, ICs=immune cells, CPS=combined positive score


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